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**Possible pathologic effect of HMGB1 on
P. gingivalis induced inflammatory response
by macrophages.**

Michelle M. Torres Santos, D.M.D.

A Thesis Presented to the Faculty of the College of Dental Medicine of Nova Southeastern
University in Partial Fulfillment of the Requirements for the Degree of
MASTER OF SCIENCE

July 2019

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Possible pathologic effect of HMGB1 on *P. gingivalis* induced inflammatory response by macrophages.

By

Michelle M. Torres Santos, D.M.D.

A thesis submitted to the College of Dental Medicine of Nova Southeastern

University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Periodontics

College of Dental Medicine

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July 2019

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I certify that I am the sole author of this thesis, and that any assistance I received in its preparation has been fully acknowledged and disclosed in the thesis. I have cited any sources from which I used ideas, data, or words, and labeled as quotations any directly quoted phrases or passages, as well as providing proper documentation and citations. This thesis was prepared by me, specifically for the M.S. degree and for this assignment.

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DEDICATION

I dedicate this to my husband, Rohit Mathur, for his unconditional support and encouragement throughout the past three years of my post-graduate journey. Thank you for celebrating with me every single little accomplishment, thank you for wiping my tears when I was making big deals out of nothing, but most importantly, thank you for always believing in me and always encouraging me to challenge myself and strive for my dreams.

I also want to dedicate this to all my family, friends and everyone that one way or another contributed to this great achievement and supported me either morally, financially or physically. The completion of this thesis could not have been possible without the unwavering support of my parents Maricarmen Santos and Vladimir Rivera, and my grandfather Hector Santos. I am eternally grateful for your unconditional love and for being my personal cheerleaders in every academic and personal endeavor.

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ABSTRACT

Possible pathologic effect of HMGB1 on *P. gingivalis* induced inflammatory response by macrophages.

DEGREE DATE: July 2019

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BACKGROUND: Periodontitis is an inflammatory disease caused by poly-microbial infection that leads to destruction of connective tissue and alveolar bone. It is well documented that bacteria-derived virulent factors that can act on Toll-like receptors (TLRs), represented by Lipopolysaccharides (LPS), are engaged in the initiation of inflammatory responses. However, while LPS is also produced by the bacteria colonized in the healthy periodontal tissue, inflammation is not induced by LPS in those periodontal healthy subjects, suggesting the requirement of additional factor to upregulate the LPS-mediated inflammatory response in periodontal tissue. Recent studies revealed that novel class of endogenous proinflammatory mediator, high mobility group box 1 (HMGB1) protein, can be released extracellularly from host cells in response to a variety of stimuli, such as

pathogen invasion. Although, significantly elevated levels of HMGB1 are reported in gingival tissues and gingival crevicular fluid (GCF) of chronic periodontitis patients, its pathophysiological role in periodontitis is not clear. The herein study investigated the effects of HMGB1 on *P. gingivalis*-LPS (*Pg*-LPS)-elicited inflammation induced in macrophages using an *in vitro* assay system.

OBJECTIVE: The present study examined the effects of the novel host danger alarming molecule, high mobility group box 1 (HMGB1), on pro-inflammatory cytokine production by *P. gingivalis*-LPS (*Pg*-LPS)-stimulated macrophages.

HYPOTHESIS: We hypothesized that HMGB1 forms complex with *Pg* LPS which can induce hyper inflammatory response by macrophages. HMGB1-*Pg*-LPS complex is expected to induce production of more pro-inflammatory cytokines (i.e. Tumor Necrosis Factor alpha (TNF- α) and Interleukin 6 (IL-6)) than those induced by LPS or HMGB1 alone.

MATERIALS AND METHODS: RAW264.7 macrophage cells (ATCC) were stimulated *in vitro* for 24 hours with HMGB1, *P. gingivalis*-LPS, *E. coli*-LPS or HMGB1 in a preformed complex with *P. gingivalis*-LPS or *E. coli*-LPS. Supernatants were collected and kept at -20°C until analysis. Chemical antagonists for TLR4, TLR2 and RAGE were applied to some cultures in the presence or absence of LPS and/or HMGB1. TNF- α and IL-6 levels in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA). PAMP receptor genes analysis was performed to assess TLR2, TLR4 and RAGE mRNAs expressed in RAW264.7 cells by quantitative PCR technique. The levels of

macrophage (RAW264.7) proliferations in response to stimulation to LPS with or without HMGB1 was determined by WST assay.

RESULTS: Compared to *E.coli*-LPS that induced prominently elevated TNF- α production by RAW264.7 macrophages, *Pg*-LPS as well as HMGB1 showed significantly lower levels of TNF- α production. However, combination of HMGB1 with *Pg*-LPS, but not *E.coli*-LPS, showed a remarkable additive effect on TNF- α production by RAW264.7-macrophages which was abrogated by addition of TLR4-antagonist. Interestingly, additive effect was only found on production of TNF- α , but not IL-6. HMGB1-*Pg*-LPS complex also increased the proliferation of macrophages, whereas HMGB1-*E.coli*-LPS complex did not affect the proliferation of macrophages. According to the qPCR-based analysis of gene expressions for PAMP receptors, the macrophages used in this study expressed mRNAs for TLR2, TLR4 and RAGE as putative ligands for HMGB1. *Pg*-LPS alone or in combination with HMGB1 did not change the expression levels of all three PAMPs expressed by macrophages, indicating that elevated production of TNF- α by HMGB1-*Pg*-LPS complex was not mediated by modulation of PAMP receptors expressed on macrophages.

CONCLUSION: These results demonstrated that HMGB1 can form a hyper-inflammatory complex with *Pg*-LPS, but not *E. coli*-LPS, that activates TLR4 and promotes TNF- α production from the macrophages, suggesting that locally released HMGB1 may up-regulate the pathogenic engagement of keystone pathogen, *P. gingivalis*, in periodontitis.

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LIST OF ABBREVIATIONS

RAGE	Receptor for advanced glycation end products
DMEM	Dulbecco's modified eagle medium
PAMP	Pathogen-associated molecular patterns
DAMP	Damage-associated molecular patterns
TLR	Toll-like receptors
HMGB1	High mobility group box 1
TNF-α	Tumor Necrosis Factor-alfa
IL	Interleukin
IL-1β	Interleukin-1 beta
LPS	Lipopolysaccharide
<i>Pg</i>	<i>Porphyromonas gingivalis</i>
<i>E. coli</i>	<i>Escherichia coli</i>
GCF	Gingival crevicular fluid
PICF	Peri-implant crevicular fluid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
qPCR	Quantitative Polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
PBS	Phosphate-buffered saline streptomycin,
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
SD	Standard deviation
ANOVA	Analysis of variance
CLP	Cecal Ligation Puncture
CD24	Cluster of Differentiation 24
CXCL12	Chemokine (C-X-C motif) ligand 1
TREM1	Triggering Receptor Expressed on Myeloid Cells 1
CXCR4	Chemokine (C-X-C motif) receptor 4
C3	Complement Component 3

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND

1.1.1 Bacterial pathogen associated molecular patterns (PAMPs)

Bacterial pathogen associated molecular patterns (PAMPs) are produced by bacteria from the plaque of periodontitis as well as periodontally healthy plaque.¹ The current consensus of periodontitis pathogenesis supports that PAMPs, such as LPS by bacteria, play a key role in induction of inflammation by binding to Toll like receptors (TLRs) expressed on the host innate immune cells.² However, LPS is also produced by bacteria present in the periodontal plaque of healthy subjects in which does not induce inflammation.¹ While consensus supports that the theory of endotoxin-tolerance may account for the healthy subject's unresponsiveness to LPS, it remains elusive how periodontal inflammation of patients with periodontitis is induced in the state of previously acquired endotoxin-tolerance.³ It is plausible that, besides PAMPs, there may be additional factor or mechanism that modulate the inflammatory responses elicited by bacterial challenge in periodontitis.

1.1.2 HMGB1 - an endogenous damage-associated molecular patterns (DAMPs)

HMGB1, an endogenous damage-associated molecular patterns (DAMPs), forms hyper inflammatory complex with PAMPs. High mobility group box 1 (HMGB1) protein was originally discovered as a nuclear factor that plays a relevant role in regulation of transcription by binding DNA and transcription factors. Therefore, it was believed that HMGB1 only exists within the nucleus to

act as a nuclear DNA chaperone exerting vital functions. However, emerging studies revealed that extracellularly released HMGB1 functions as a pro-inflammatory factor and that it is correlated with multiple inflammatory and autoimmune diseases.^{4,5} When tissue injury is inflicted, HMGB1 can be released extracellularly conveying several inflammatory functions.⁶ Based on its nature released from endogenous cells in response of tissue injury, HMGB1 belongs to DAMPs, also known as alarmins. HMGB1 can be actively secreted into the extracellular space by activated monocytes and macrophages, or passively released from the nuclei of necrotic or damaged cells. Extracellularly released HMGB1 binds Receptor for Advanced Glycation End-products (RAGE) to elicit the inflammatory signal which, in turn, lead to the production of proinflammatory factors.^{7,8} Very importantly, HMGB1 forms a hyper inflammatory complex with bacterial LPS or IL-1 and stimulates inflammation by binding with TLR2, TLR4 and IL-1R. For example, it is reported that stimulation with preformed HMGB1-LPS complexes induces a 100-fold stronger response than stimulation with comparable levels of LPS alone. Thus, HMGB1 acts as a key pro-inflammatory factor and late-acting distal inflammatory mediator during cell injury necrosis and inflammation.⁹ Compared with other pro-inflammatory cytokines, the release of HMGB1 is retarded for several hours and it can be promoted by IL-1 β and TNF- α . Subsequently, extracellular HMGB1 convey a positive feedback loop by activating the monocytes to stimulate further release of pro-inflammation cytokines.¹⁰

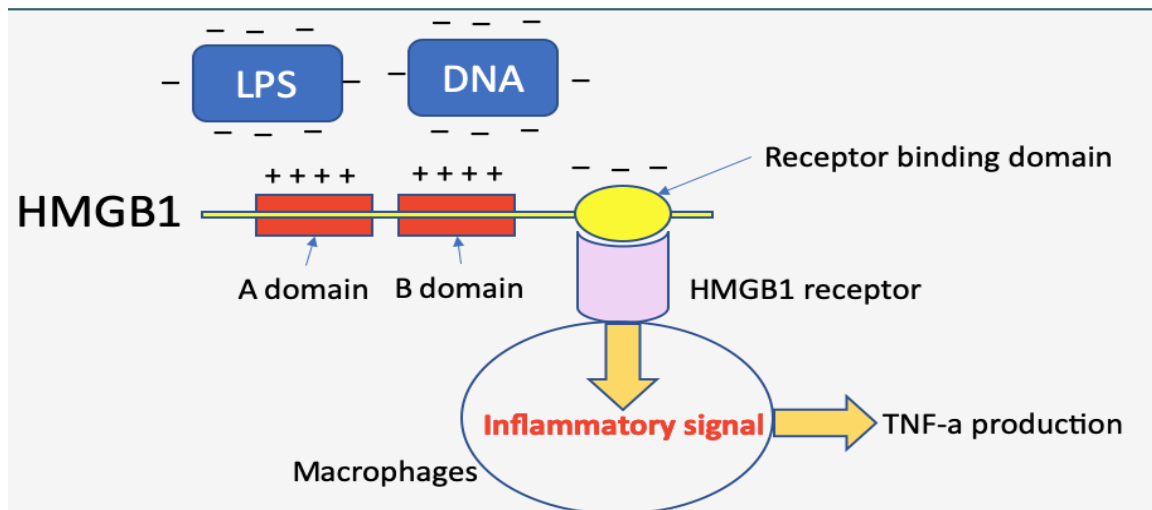


Figure 1: Extracellular HMGB1's property to adhere PAMPs. HMGB1 is a dynamic protein composed of three domains. Positively charged domain A/B binds to negatively charged DNA and LPS. Negatively charged receptor binding domain facilitates its ligation to HMGB1 receptors

1.1.5 Extracellularly released HMGB1 in Periodontal Diseases

The level of extracellularly released HMGB1 are elevated in periodontitis.¹¹ In the oral cavity, lipopolysaccharide (LPS) of major periodontal pathogens, such as *Porphyromonas gingivalis* (*Pg*), induces HMGB1 secretion from human gingival fibroblasts which might contribute to periodontal tissue destruction.^{3,10,12} Significantly elevated levels of HMGB1 are reported in gingival tissues and GCF of chronic periodontitis patient, as compared to periodontally healthy patients.^{12,13} Expression of HMGB1 in peri-implant crevicular fluid (PICF) is also elevated in the patients with peri-implantitis.⁹ Elucidating HMGB1 receptor usage in processes where HMGB1 acts alone or in complex with other molecules

in the context of periodontitis is essential for the understanding of basic HMGB1 biology and for designing HMGB1-targeted therapies.

1.1.4 *Porphyromonas gingivalis* – Microbiology and Pathogenesis

It was originally reported that *P. gingivalis* LPS can bind with TLR2, unlike to other Gram negative bacterial LPS that bind with TLR4. However, later studies revealed that *Pg*-LPS activates cells through binding with TLR4, but not TLR2. The contaminant lipoprotein in *Pg*-LPS samples was attributed to the previously reported *Pg*-LPS' activity to stimulate TLR2.¹⁴ It is thought that pathogenesis of periodontitis is elicited by “dysbiosis of microbiome” in periodontal plaque resulting from the diminished number of beneficial symbionts and inversely increased number of pathogens, such as *P. gingivalis*, a Gram-negative anaerobe, possesses unique feature compared to the other Gram-negative bacteria.^{15,16} Especially, LPS derived from *P. gingivalis* has been shown to differ from LPS produced by other Gram-negative bacteria in structure and function.¹⁶ The number of *P. gingivalis* identified in the dental plaque of periodontal pocket is proportional to the levels of pro-inflammatory cytokines detected in gingival crevice fluid (GCF), indicating that unique virulent factor produced by *P. gingivalis* is engaged in the induction and/or upregulation of inflammatory response in periodontitis.^{1,2} However, the virulent factor produced by *P. gingivalis* that is responsible for the upregulation of pro-inflammatory cytokine production in periodontitis remains elusive.

1.1.5 HMGB1 and *P. gingivalis*-LPS

In terms of unique property of HMGB1 that forms hyper reactive complex with LPS and other bacterial PAMPs,¹⁷ it is not known whether *P. gingivalis* LPS can form a complex with HMGB1 and if such HMGB1-*Pg*-LPS complex upregulates the inflammatory signal as a result of ligation with, TLR4, TLR2 or RAGE. The possible role and mechanisms by which HMGB1 is involved in regulating inflammatory response of periodontitis in association with *P. gingivalis* infection is largely unknown. This is the first study to compare the difference between *P. gingivalis* LPS-HMGB1 complex and *E. coli* LPS-HMGB1 complex in induction of inflammatory response by innate immune macrophages. Findings from this investigation not only elucidated the basic pathophysiology of HMGB1 as a pro-inflammatory promoter for bacterial PAMPs, but also identified the molecular target to design a novel therapeutic approach for periodontitis.

1.2 CURRENT STUDY

1.2.1 Purpose

Since periodontitis not only deteriorates the patient's quality of life, but also increases the risk for chronic systemic diseases, there is a strong need for developing novel preventive and therapeutic approaches for this disease. Therefore, the present study that challenges to elucidate the pathogenesis of periodontitis has translational significance.

The current consensus of periodontal pathogenesis supports that pathogen associated molecular patterns (PAMPs), such as LPS released from

bacteria, play a key role in induction of inflammation by binding to Toll like receptors (TLRs) expressed on the host innate immune cells. However, LPS is produced by bacteria present in the periodontal plaque of healthy subjects which don't induce inflammation, indicating that there may be additional factors that modulate the inflammatory responses elicited by bacterial challenge in periodontitis. To this end, the purpose of the present study was to investigate the role of danger associated molecular pattern (DAMPs), especially, HMGB1, as a TLR-signal modulatory factor, by forming a hyper inflammatory complex with PAMPs. It is plausible that the proposed study may elucidate the molecular mechanism that can't be explained by simple PAMPs-TLR inflammation axis, possibly leading to the paradigm-shift in the research of periodontal pathogenesis.

1.2.2 Hypothesis

We hypothesized that HMGB1 forms complex with *Pg* LPS which can induce hyper inflammatory response by macrophages. HMGB1-*Pg*-LPS complex is expected to induce production of more pro-inflammatory cytokines (i.e. Tumor Necrosis Factor alpha (TNF- α) and Interleukin 6 (IL-6)) than those induced by LPS or HMGB1 alone

1.2.3 Specific Aims

To address the above noted hypothesis, the specific aims of this study is to understand the possible effects of HMGB1, a danger associated molecular pattern (DAMP), on the *P. gingivalis* LPS-induced inflammatory response by

macrophages. We were in particular interested to compare the magnitude of inflammation induced by complex between HMGB1 and *P. gingivalis* LPS, compared to that between HMGB1 and *E. coli* LPS. We also challenged to identify the receptors that are responsible for binding the *Pg*-LPS/HMGB1 complex.

Specific Objectives:

Specific Aim 1: To assess the production pattern of pro-inflammatory cytokines (TNF- α , IL-6) by a mouse macrophage cell line (RAW264.7) in response to the stimulation with *P. gingivalis* LPS or *E. coli* LPS in the presence or absence of recombinant HMGB1. The expressions of PAMP receptors (TLR2, TLR4, and RAGE) mRNA and levels of the aforementioned cytokines (TNF- α , IL-6) were monitored using qPCR and ELISA.

Specific Aim 2: To determine the receptor which is responsible for the ligation of HMGB1/*Pg*-LPS complex and to induce inflammatory signal. RAW264.7 cells were stimulated with HMGB1/*Pg*-LPS complex in the presence or absence of chemical antagonist of TLR2, TLR4, and RAGE, and the expression patterns of TNF- α were compared using ELISA.

CHAPTER 2: MATERIALS AND METHODS

2.1 SAMPLING PLAN

2.1.1 Sample Size

For a power of 0.8 and alpha of 0.05, experiments of cell culture and sampling were performed using triplicate wells or more for each treatment. As a typical example of data collected from TNF- α produced by mouse macrophage cell line (RAW264.7 – 10^4 cells/well) stimulated with or without LPS, average \pm SD for no stimulation is 1.0 ± 0.17 ng/ml, while that of LPS-stimulated group is 1.4 ± 0.17 . Based on the power calculation, $n=3$ /group was required.

2.2 INSTRUMENTATION

2.2.1 Dependent Variables

Cytokine analysis: TNF- α and IL-6 (ng/mL) levels in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA).

Gene Expression: Quantitative PCR were conducted to measure TLR2, TLR4 and RAGE genes (relative ratio compared to GAPDH). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as internal control.

2.2.2 Independent Variables

Inhibitors for TLR4, TLR2 and RAGE (10, 100 ng/ml, respectively) were applied to some cultures in the presence or absence of *P. gingivalis* LPS alone, *E. coli*

LPS alone, HMGB1 alone, *E. coli* LPS-HMGB1 complex, and *P. gingivalis* LPS-HMGB1 complex.

2.2.3 Levels of Measurements

Variables were quantitative and continuous. RAW264.7 macrophages cells were stimulated with HMGB1 and the effects on cytokine production and mRNA expressions were evaluated using ELISA and quantitative Polymerase Chain Reaction (PCR), respectively.

Reliability and Validity: All experiments were performed in a triplicate fashion and repeated at least twice to confirm the reproducibility.

2.3 STUDY DESIGN

2.3.1 Overall Experiment Design

RAW264.7 macrophage cells (ATCC) were plated at 100,000 cells per well in a 96-well plate in culture medium (DMEM medium supplemented with 10% FBS, penicillin, streptomycin, HEPES, sodium pyruvate and β -mercaptoethanol). Cells were then stimulated for 24 hours with HMGB1, *P. gingivalis* LPS, *E. coli* LPS or HMGB1 in a preformed complex with *P. gingivalis* LPS or *E. coli* LPS under standard culture conditions. Supernatants were collected to perform various analyses. Proinflammatory cytokines, TNF- α and IL-6, levels in the culture supernatants were measured by ELISA. The levels of macrophage (RAW264.7) proliferations in response to stimulation with LPS in the presence or absence of HMGB1 was determined by WST assay. PAMP receptor

genes analysis of TLR2, TLR4 and RAGE mRNAs expressed in RAW264.7 cell culture were measured by quantitative PCR technique.

The second purpose of the experiment was to determine the receptor(s) engaged in the recognition of complex generated between HMGB1 and *E. coli*-LPS, or Pg-LPS. To this end, chemical antagonists for TLR4, TLR2 and RAGE (10, 100 ng/ml, respectively) were applied to the RAW264.7 cell cultures in the presence or absence of *Pg*-LPS and/or HMGB1. After stimulation for 24 hours, TNF- α levels in the culture supernatants were measured by ELISA.

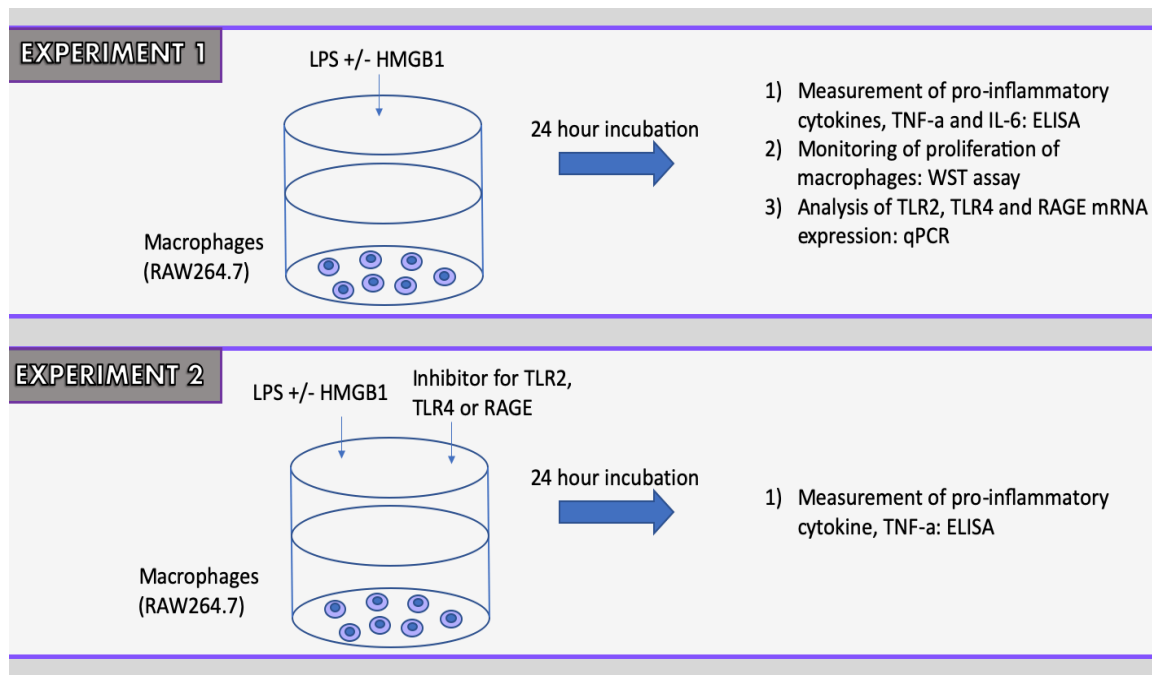


Figure 2: Overall Experiment Design

2.3.2 Reagents

Tag-free recombinant rat HMGB1 (free of endotoxin) was purchased from Creative Biomart (Shirley, NY, 99% homology to human and mouse HMGB1). Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS),

penicillin, streptomycin, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), sodium pyruvate and β -mercaptoethanol were from Invitrogen (Carlsbad, CA, USA). Highly purified LPS extracted from *Escherichia coli* O55:B5 and *P. gingivalis* were also purchased from Invivogen (San Diego, CA, USA). Chemical antagonists for TLR4, TLR2 and RAGE, i.e., Resatorvid (TAK-242), 3,4,6-Trihydroxy-2-methoxy-5-oxo-5H-benzocycloheptene-8-carboxylic acid hexyl ester (CU-CPT22) and N-Benzyl-4-chloro-N-cyclohexylbenzamide (FPS-ZM1), respectively, were purchased from Calbiochem.

2.3.3 Culture of Macrophages

RAW264.7 mouse macrophage cells (ATCC) were plated at 100,000 cells per well in a 96-well plate in DMEM medium supplemented with 10% FBS, penicillin, streptomycin, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), sodium pyruvate and β -mercaptoethanol. Cells were then stimulated for 24 hours with HMGB1, *P. gingivalis* LPS, *E. coli* LPS or HMGB1 in a preformed complex with *P. gingivalis* LPS or *E. coli* LPS. Supernatants were collected and kept at -20°C until analysis. Chemical antagonists for TLR4, TLR2 and RAGE (10, 100 ng/ml, respectively) were applied to some cultures in the presence or absence of LPS and/or HMGB1.

2.3.4 Preparation of HMGB1 Complexes

HMGB1 in PBS were mixed together with *E. coli* LPS or *P. gingivalis* LPS in polypropylene tubes in different ratios to give final concentrations in cell cultures. The stimulatory ability of the complexes depends on both the

temperature and the length of time in which they are formed. For this study, the mixtures were incubated at room temperature (25°C) for 10 minutes before addition to the cell culture, following the protocol published by another group.^{18,19}

2.3.5 Cytokine Analysis

TNF- α and IL-6 levels in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) using DuoSet kits from R&D Systems (Minneapolis, MN, USA) and following the manufacturer's instructions. Mouse HMGB1 ELISA kit were purchased from LifeSpan BioScience inc (Seattle, WA, USA).

2.3.6 Polymerase Chain Reaction (PCR)

mRNAs for TLR2, TLR4 and RAGE expressed in RAW264.7 cells were measured by quantitative PCR technique. Total RNA was isolated from cells using RNA-Bee isolation reagent (AMS Biotechnology, Cambridge, MA, USA), following the manufacturer's instructions, and subjected to reverse transcription with the Verso cDNA synthesis kit (Thermo Fisher, Waltham, MA, USA) in the presence of random primers and oligo-dT. Gene expression was quantified using the LightCycler® 480 SYBR Green I Master (Roche Diagnostics, Indianapolis, IN, USA). The list of Primer (Taqman, Applied Biosystems inc):

- mouse TLR2; Mm01213946_g1 Tlr2
- mouse TLR4; Mm00445273_m1 Tlr4
- mouse RAGE; Mm01134790_g1 Ager
- Control GAPDH; Mm 99999915 g1 Gapdh

2.3.7 WST PROLIFERATION ASSAY

Following the culture method described above, RAW264.7 cells were incubated in a 96 well plate (100,000 cells/200 ul/well) with test compounds (LPS or HMGB1) for 24 hours. After the incubation, 100 ul of culture supernatant was removed and the final volume of culture medium in each well was adjusted to 100 μ l. Then, Cell Proliferation Reagent WST-1 (10 ul/well, Millipore Sigma) was added to each well of 96-well plate. The plates were incubated at 37°C for 2 hours. The developed color in each well of the plate was measured for the absorbance at optical density (OD) 450 nm using a plate reader (Synergy H1, Biotek).

2.4 STATISTICAL ANALYSIS

The proposed *in vitro* experiments were performed in a triplicate fashion and repeated at least twice to confirm the reproducibility. Descriptive statistics were calculated for all study variables. This includes the mean and standard deviation (SD) for continuous measures, counts and percentages for categorical variables. A Welch, two-samples t-test, was used to compare means between the control and experimental groups. To look for differences between more than two groups, a general linear model (ANOVA) with robust standard errors was used. Tukey's HSD test was employed for all post-hoc comparisons. The statistical package R 3.2.2 was used to create and test the ANOVA models. Statistical significance is found at $p < 0.05$.

CHAPTER 3: RESULTS

3.1. PRO-INFLAMMATORY CYTOKINES ANALYSIS: ELISA

The inflammatory responses of all five groups were assessed by measuring the levels of proinflammatory cytokines produced by macrophage (RAW 264.7) cells. Supernatants collected from the RAW 264.7 cells stimulated with or without LPS and/or HMGB1 for 24 hours were subject to commercially available enzyme-linked immunosorbent assay (ELISA) for TNF- α and IL-6. Results from measuring TNF- α levels showed that *E. coli*-LPS either alone or in combination with HMGB1 complex induced prominently elevated TNF- α production compared to the stimulation with HMGB1 alone. Although addition of HMGB1 to all three concentrations of LPS promoted inflammatory reaction of TNF- α production by RAW 264.7 cells, such a combination of HMGB1 with *E. coli*-LPS showed very modest additive effects (about 20% increase). (Figures 3 and 5).

Among various concentrations (10, 100 and 1000 ng/ml) of three different simulants tested (*E. coli*-LPS, *Pg*-LPS, and HMGB1), the pro-inflammatory effect of HMGB1 or *Pg*-LPS in induction of TNF- α production by RAW264.7 macrophages were significantly lower than that mediated by *E. coli*-LPS. More specifically, compared to *E. coli*-LPS that induced prominently elevated TNF- α production by RAW264.7-macrophages (4000 – 6000 pg/ml), *Pg*-LPS as well as HMGB1 displayed significantly lower levels of TNF- α production (at most 1000

pg/ml). However, combination of HMGB1 with *Pg*-LPS, but not *E. coli*-LPS, showed a remarkable synergistic pro-inflammatory effect on levels of TNF- α release when added to cultures of RAW264.7- macrophages. (Figures 4 and 5).

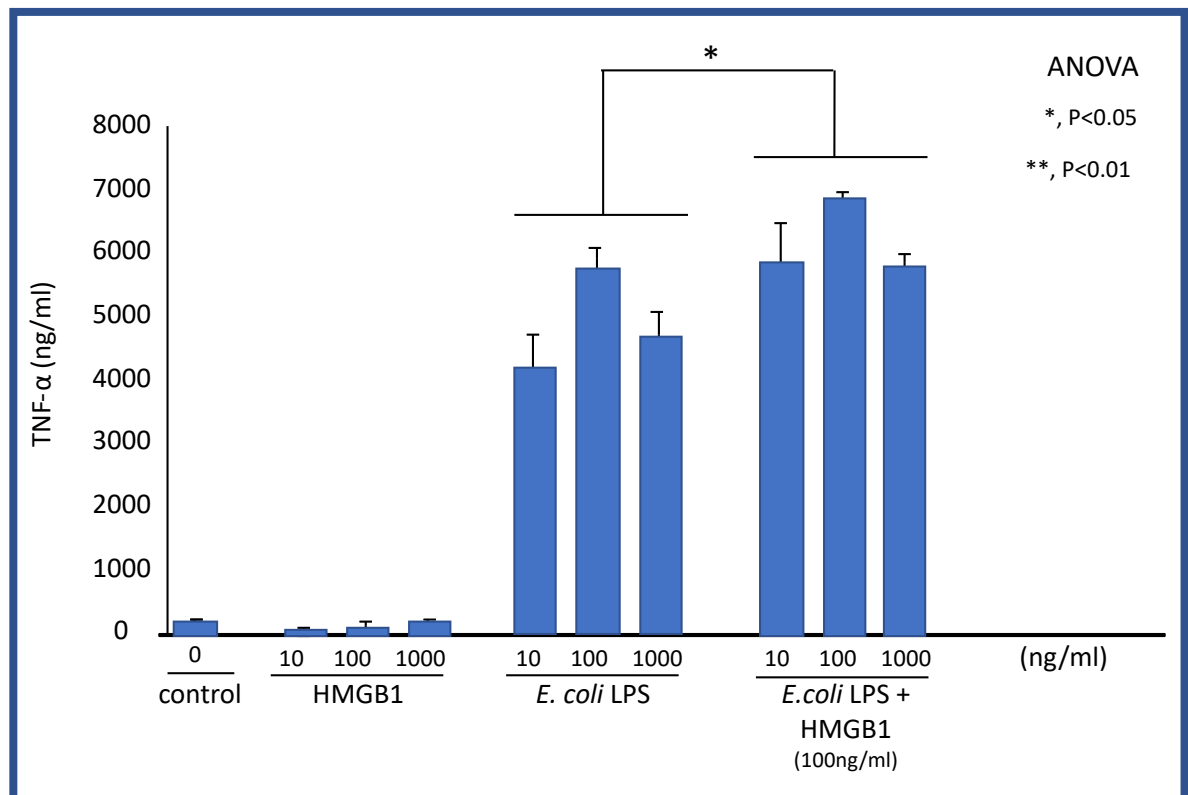


Figure 3: Inflammatory response determined by TNF- α production from RAW264.7-macrophages stimulated with HMGB1, *E. coli*-LPS or HMGB1/*E. coli*-LPS complex. *E. coli*-LPS alone induced prominently elevated TNF- α production, while its complex in combination with HMGB1 showed very modest (about 20%) increase of pro-inflammatory responses.

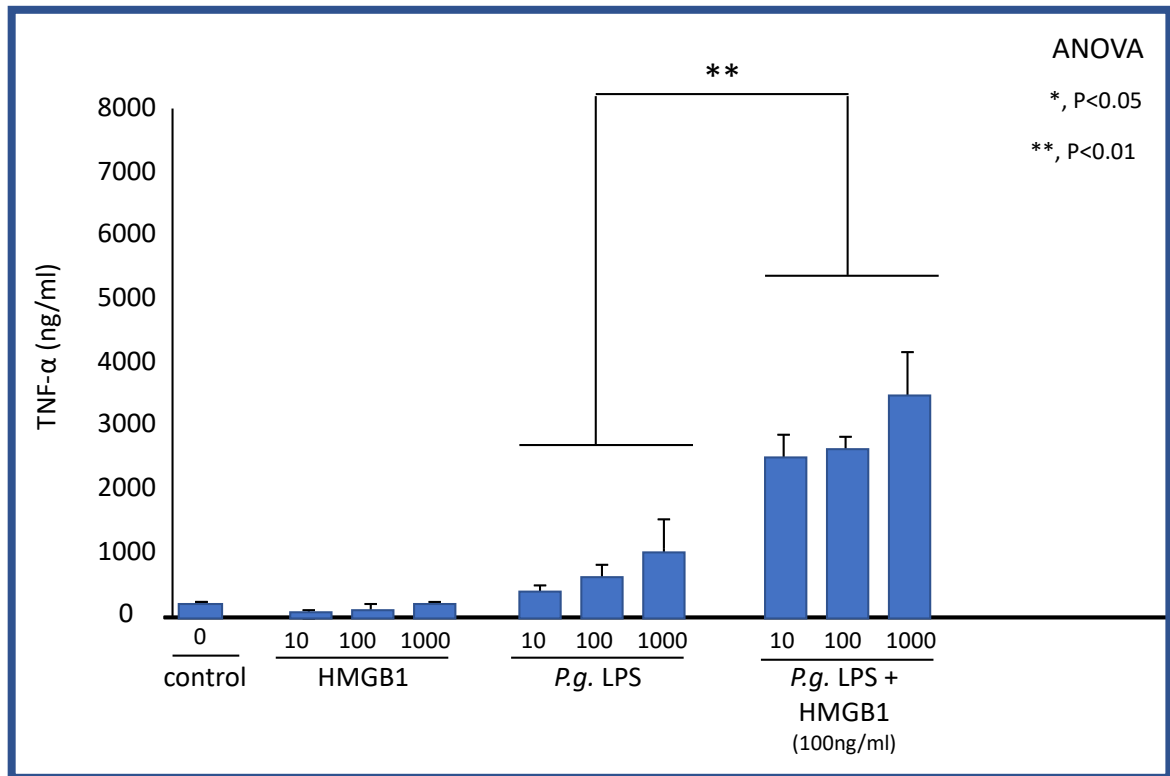


Figure 4: Inflammatory response based on TNF- α production by RAW264.7-macrophages following stimulation with HMGB1, *Pg*-LPS, or HMGB1/*Pg*-LPS complex. HMGB1 produced higher additive effects in combination with *Pg*-LPS, than with *E. coli*-LPS.

Although both combinations of *E. coli*-LPS and HMGB1 as well as *Pg*-LPS and HMGB1 promoted the increased inflammatory reaction of TNF- α , the magnitude of HMGB1-induced synergistic effects with *Pg*-LPS indicates that HMGB1 can form a hyper inflammatory complex with *Pg*-LPS, but not *E. coli*-LPS. The combination of HMGB1 with *Pg*-LPS induced remarkably stronger pro-inflammatory response than the response induced by HMGB1/*E.coli*-LPS complex. Indeed, the combination between HMGB1 and *E. coli*-LPS showed very modest additive effect (about 20%) compared to *E. coli*-LPS alone (Figure 5).

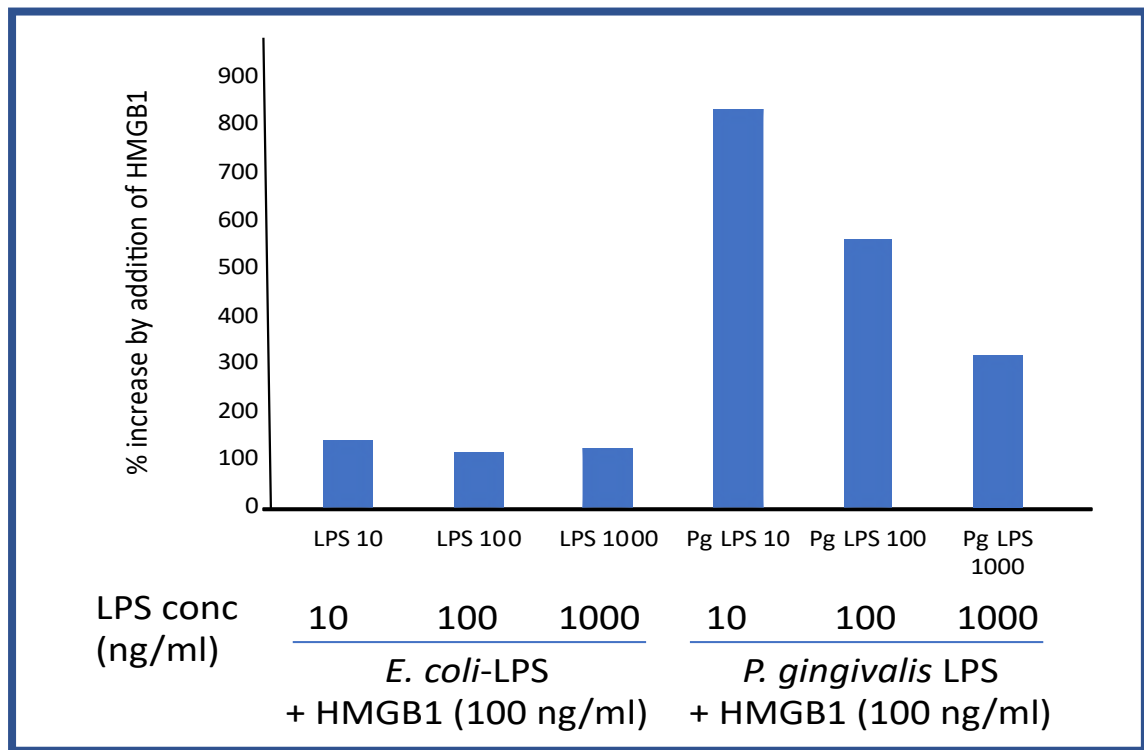


Figure 5: Percentage of increased TNF- α production by the addition of HMGB1.

In comparison to *Pg*-LPS alone, at least 3-fold increase of TNF- α production was observed following RAW 264.7-cell stimulation with HMGB1/*Pg*-LPS complex (synergistic effect). Such synergistic effect found in HMGB1/*Pg*-LPS complex was not detected between *E. coli*-LPS and HMGB1 that showed 10-40% increase (additive effect).

Release of IL-6 by RAW 264.7 macrophages following the stimulation with HMGB1/*E. coli*-LPS or HMGB1/*Pg*-LPS complexes, as well as with the individual component was evaluated (Figure 6). RAW 264.7 cells failed to produce any detectable level of IL-6 or produced considerably minimal amounts of IL-6 in response to stimulation with HMGB1 alone, *P. gingivalis*-LPS alone or preformed HMGB1/*Pg*-LPS complex. *E. coli*-LPS alone and its combination with HMGB1 induced prominently elevated IL-6 production by macrophages. However, unlike

TNF- α , no additive effect by HMGB1 on *E. coli*-LPS-induced IL-6 production.

More specifically, contrast to the additive effects observed between HMGB1 and *E. coli*-LPS on TNF- α production by RAW264.7 cells, the application of preformed HMGB1 and *E. coli*-LPS displayed comparable IL-6 production (in other words, no significant difference) by RAW 264.7 macrophages.

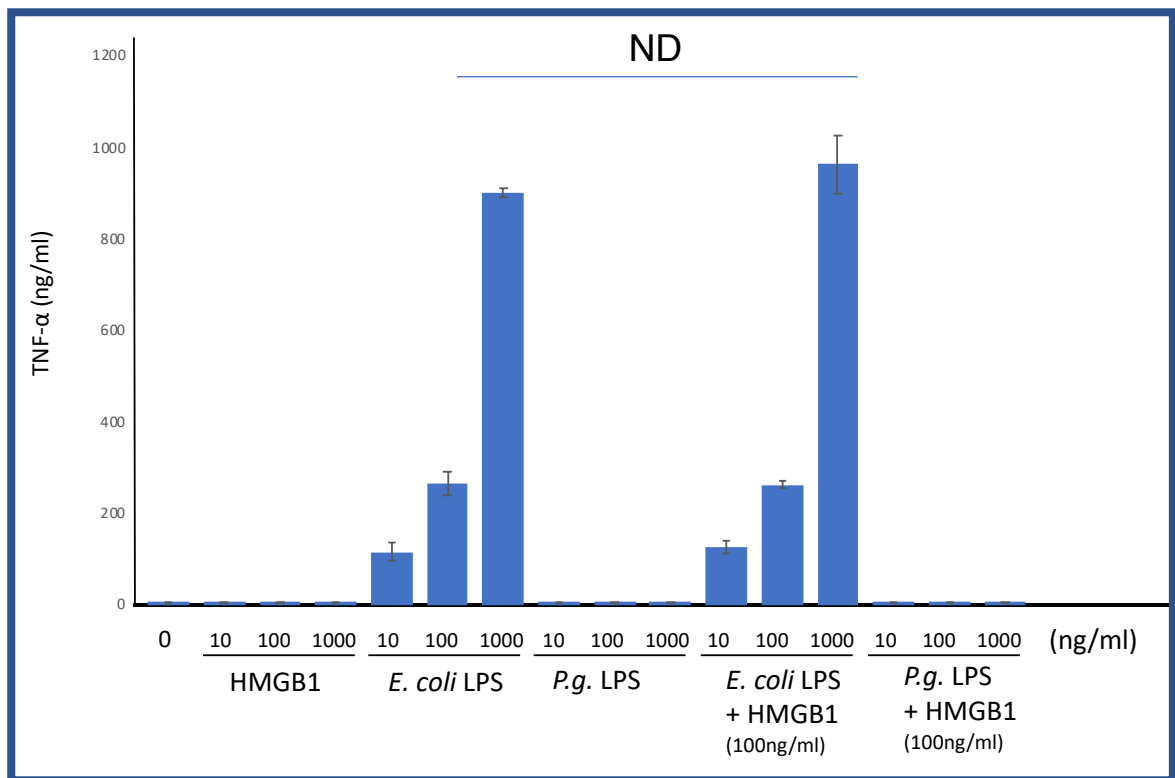


Figure 6: IL-6 production by RAW264.7-macrophages in response to stimulation with *E. coli*-LPS or *Pg*-LPS in the presence or absence of HMGB1. Stimulation with HMGB1-*Pg*-LPS complex and with the single component of HMGB1 or *Pg*-LPS failed to induce IL-6 production in macrophages. *E. coli*-LPS alone and in combination with HMGB1 induced prominently elevated IL-6 production. However, the stimulation of RAW264.7 cells with HMGB1/*E. coli* LPS complexes did not affect the IL-6 release induced by stimulation with *E. coli*-LPS alone.

3.2. MONITORING OF MACROPHAGES PROLIFERATION: WST ASSAY

To examine whether any of test compound(s) applied by itself or in combination can affect the proliferation of macrophages, WST assay was employed. After incubation of RAW264.7 cells in a 96 well plate with test compounds (LPS or HMGB1) for 24 hours, a cell proliferation reagent WST-1 was added to the culture plates and incubated at 37°C for 2 hours. Following incubation, the developed color in each well of the 96-well plate was measured. The absorbance of developed color from WST-1-reacted macrophage cells is proportional to the levels of viability and proliferation of the cells in response to stimulation with *E. coli*-LPS and *Pg*-LPS with or without HMGB1 and to HMGB1 alone. Results revealed that the preformed complex of HMGB1 in combination with *Pg*-LPS was the only group able to significantly upregulate the proliferation of RAW264.7 macrophages. HMGB1 alone, *E. coli*-LPS alone or the preformed complex of the combination between *E. coli*-LPS and HMGB1 did not affect the proliferation of macrophages, suggesting that HMGB1 combined with *Pg*-LPS elicits a unique to upregulate the proliferation of macrophages (Figure 7).

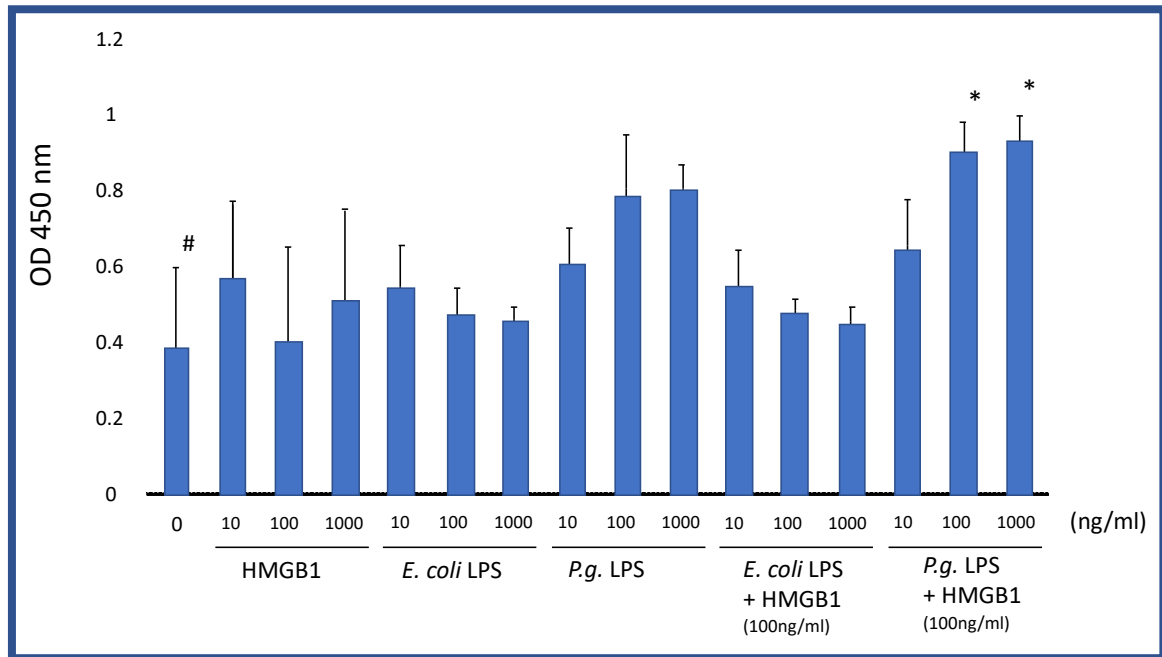


Figure 7: Determination of macrophage proliferation by the WST assay. The combination of HMGB1 and *Pg*-LPS significantly upregulated macrophage proliferation. Other tested groups did not affect the proliferation of macrophages.

3.3. ANALYSIS OF MRNA EXPRESSIONS OF PAMP RECEPTORS: qPCR

To evaluate the expression pattern of PAMP receptors genes in RAW 264.7 macrophages, expression of mRNA for TLR2, TLR4 and RAGE was analyzed using quantitative PCR. The relative ratio of the genes was compared to an internal control (GAPDH). As shown in figures 8 to 10, the macrophages used in this study expressed mRNAs for TLR2, TLR4 and RAGE, three of which are reported as putative ligands for HMGB1. Contrast to *E. coli*-LPS that significantly upregulated TLR4 mRNA expression, while suppressed both TLR2 and RAGE mRNAs, *Pg*-LPS or HMGB1 alone or in complex did not affect the expression of any mRNA of the receptor tested. Results indicated that the

additive effects between *Pg*-LPS and HMGB1 on TNF- α expression was not mediated by modulation of PAMP receptors expressed on macrophages.

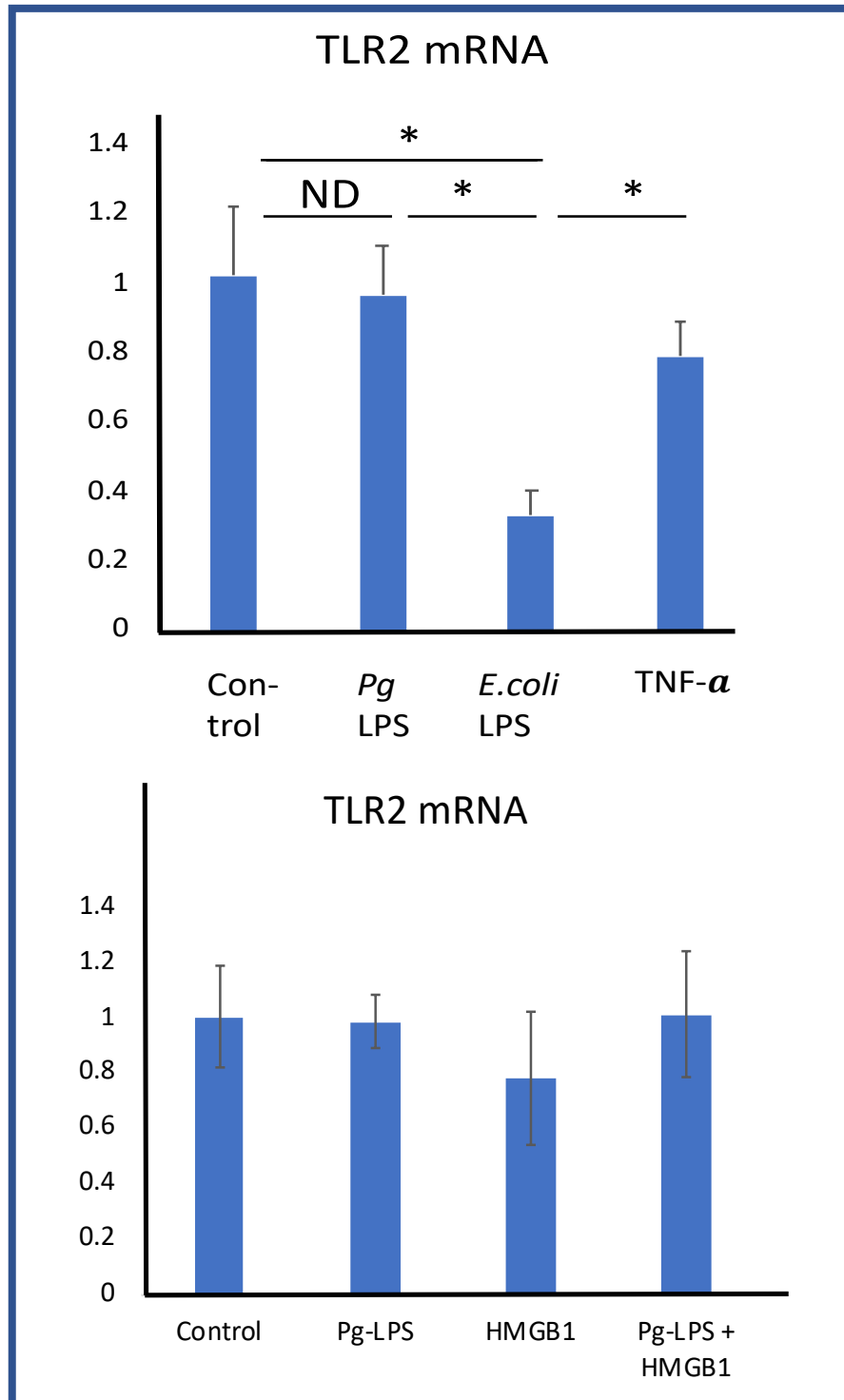


Figure 8: Expression TLR2 mRNA in RAW 264.7-macrophages.

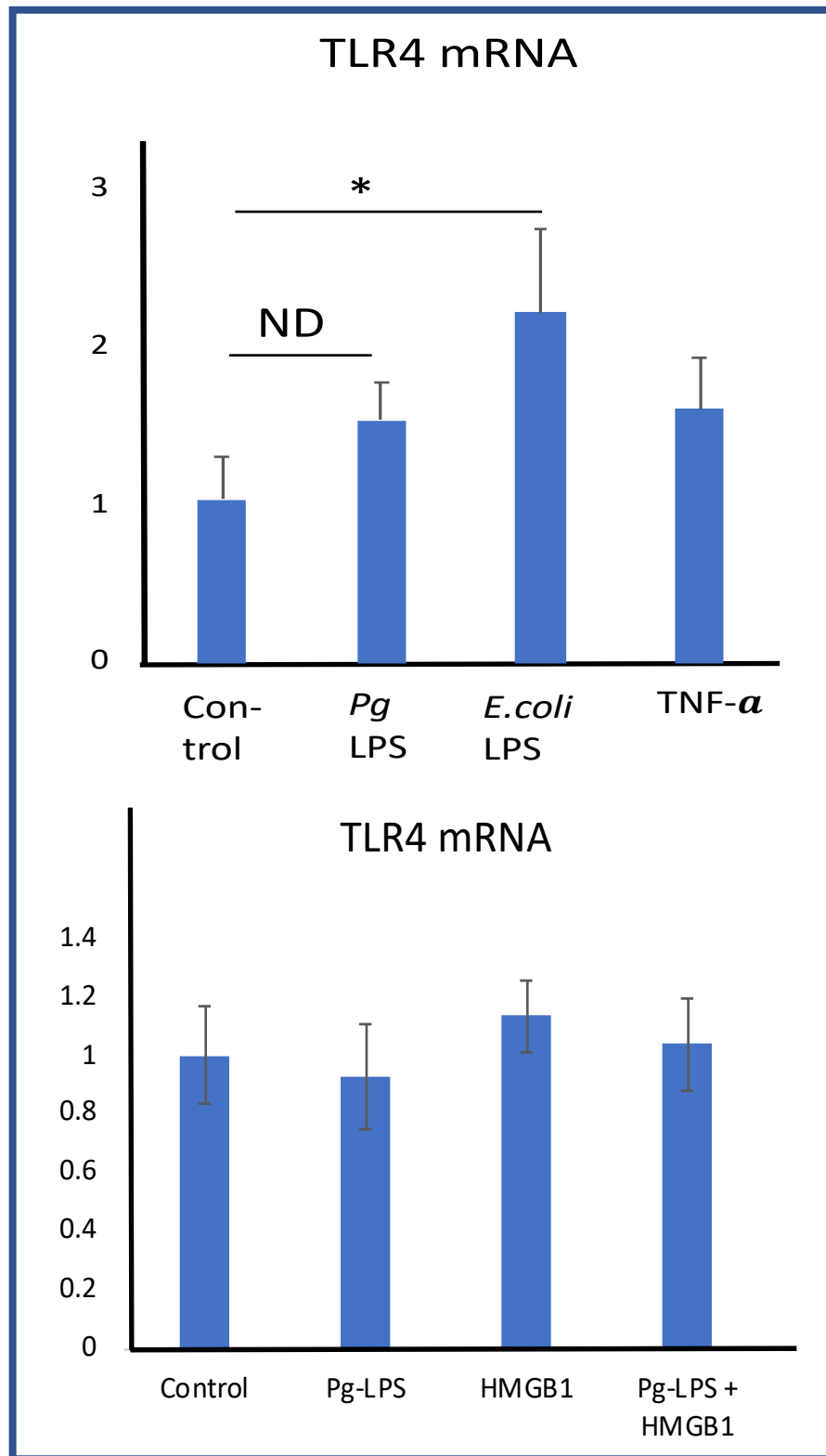


Figure 9: Expression TLR4 mRNA in RAW 264.7-macrophages.

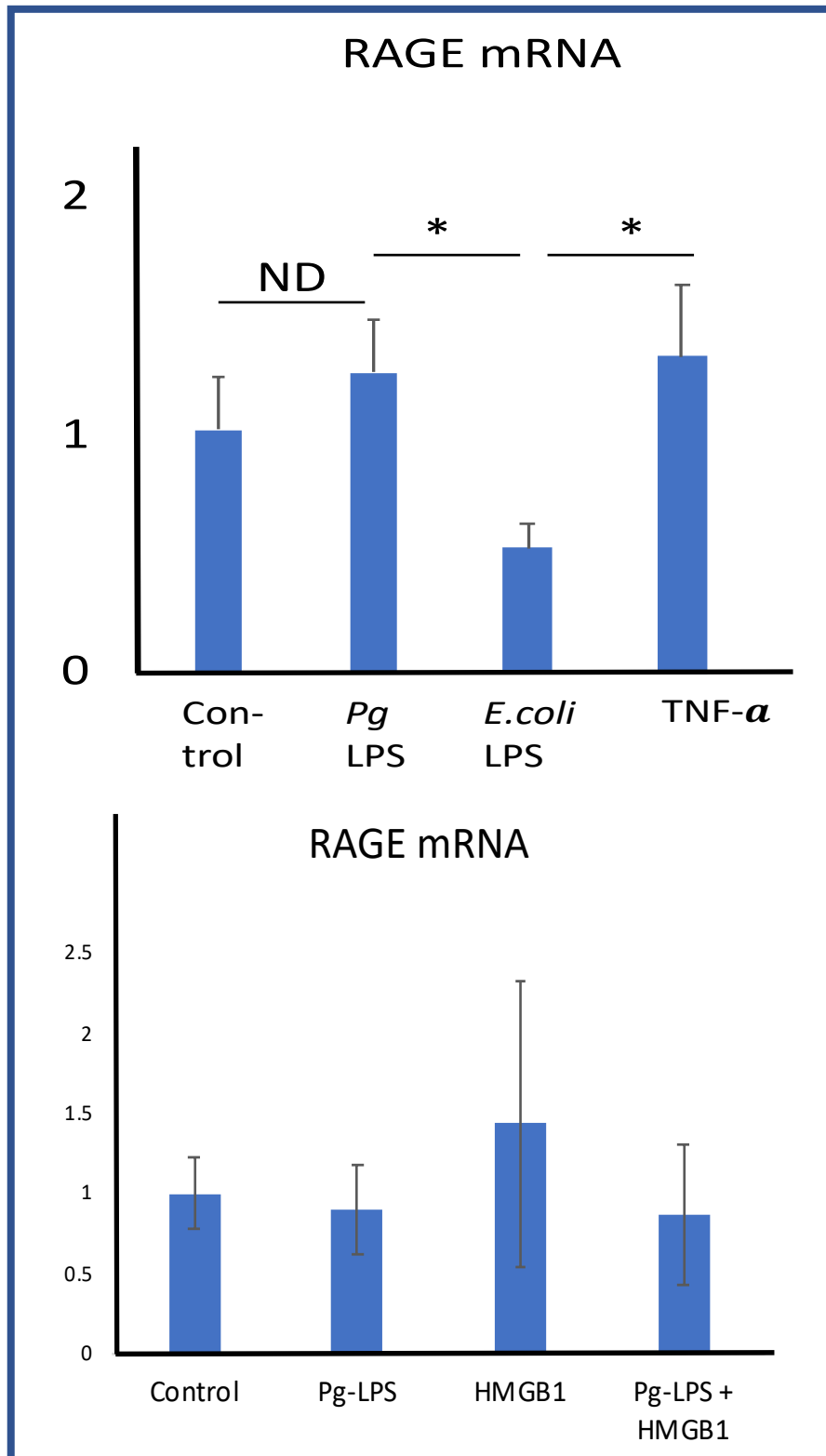


Figure 10: Expression RAGE mRNA in RAW 264.7-macrophages.

3.4. EFFECTS OF CHEMICAL INHIBITORS FOR TLR2, TLR4 AND RAGE

In order to elucidate the mechanism underlying the pro-inflammatory signaling pathways engaged in the TNF- α production by macrophages following stimulation with HMGB1 alone, *Pg*-LPS alone or the pre-formed HMGB1/*Pg*-LPS complex, chemical antagonists for TLRs and RAGE receptor were employed. TNF- α levels produced by test components-stimulated macrophage culture that also received the antagonists for TLR-2, TLR-4 or RAGE are shown in Figures 11 to 13. Results demonstrated that TNF- α secreted by macrophages stimulated with *Pg*-LPS alone was inhibited only by TLR4-inhibitor, while the TNF- α induction caused by HMGB1 alone was inhibited by both, TLR2-inhibitor and TLR4-inhibitor. Meanwhile, the synergistic effect of HMGB1-*Pg*-LPS complex that promoted TNF- α release was only inhibited by TLR4 inhibitor, but not TLR2 or RAGE inhibitor. These results indicate that HMGB1/*Pg*-LPS complex acts on TLR4, but not TLR2 or RAGE, which, in turn, upregulates the cell signal to promote the TNF- α production.

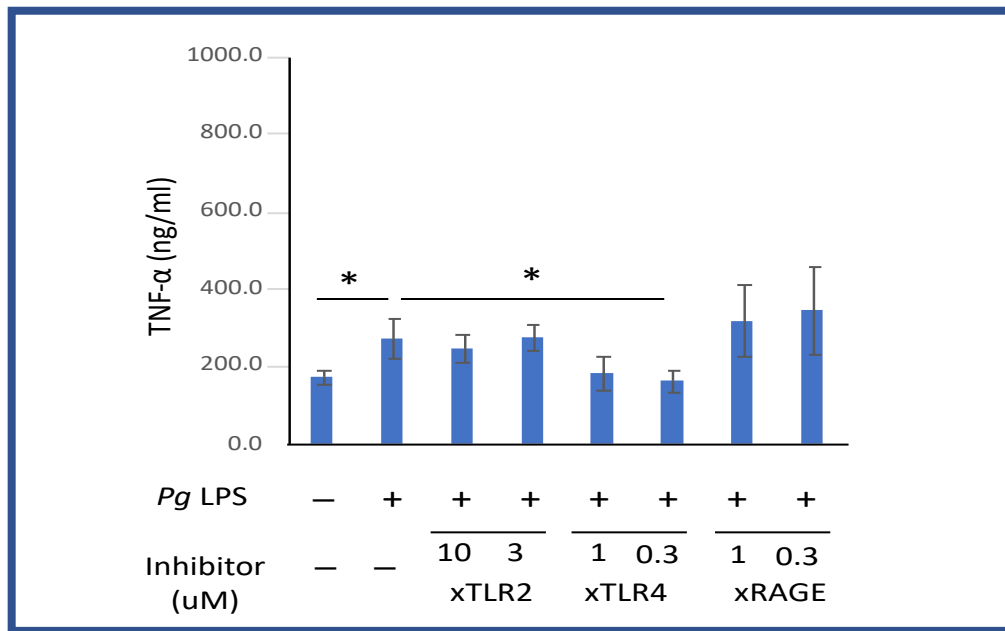


Figure 11: Effects of chemical inhibitors for TLR2, TLR4 and RAGE on TNF- α production by macrophages stimulated with *Pg*-LPS. TLR2 inhibitor: CU CPT 22 (Sigma), TL4 Inhibitor: TAK 242 (Sigma), RAGE Inhibitor: FPS-ZM1 (Sigma)

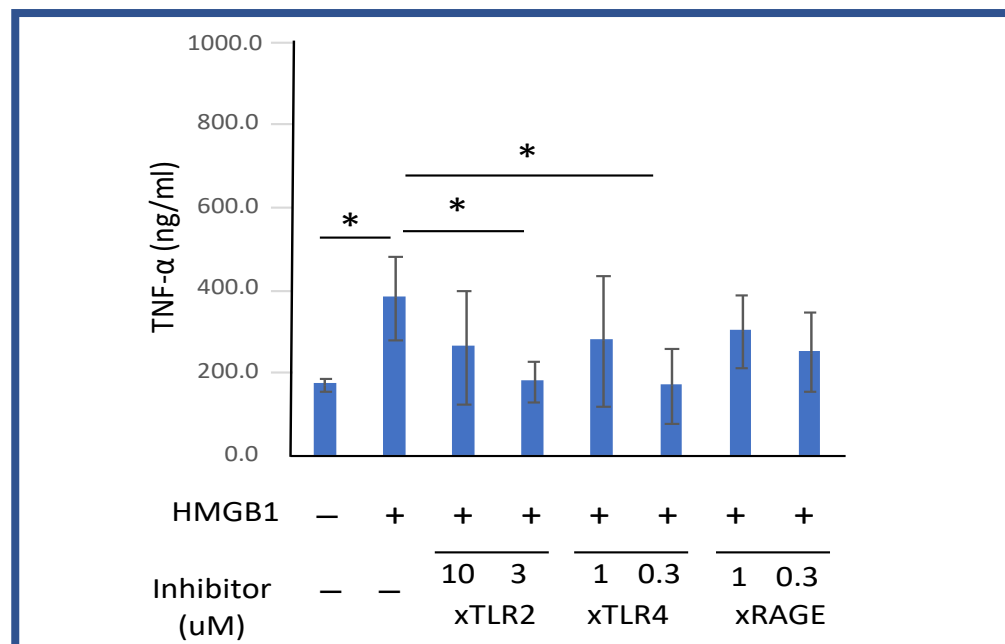


Figure 12: Effects of chemical inhibitors for TLR2, TLR4 and RAGE on TNF- α production by macrophages stimulated with HMGB1. TLR2 inhibitor: CU CPT 22 (Sigma), TL4 Inhibitor: TAK 242 (Sigma), RAGE Inhibitor: FPS-ZM1 (Sigma)

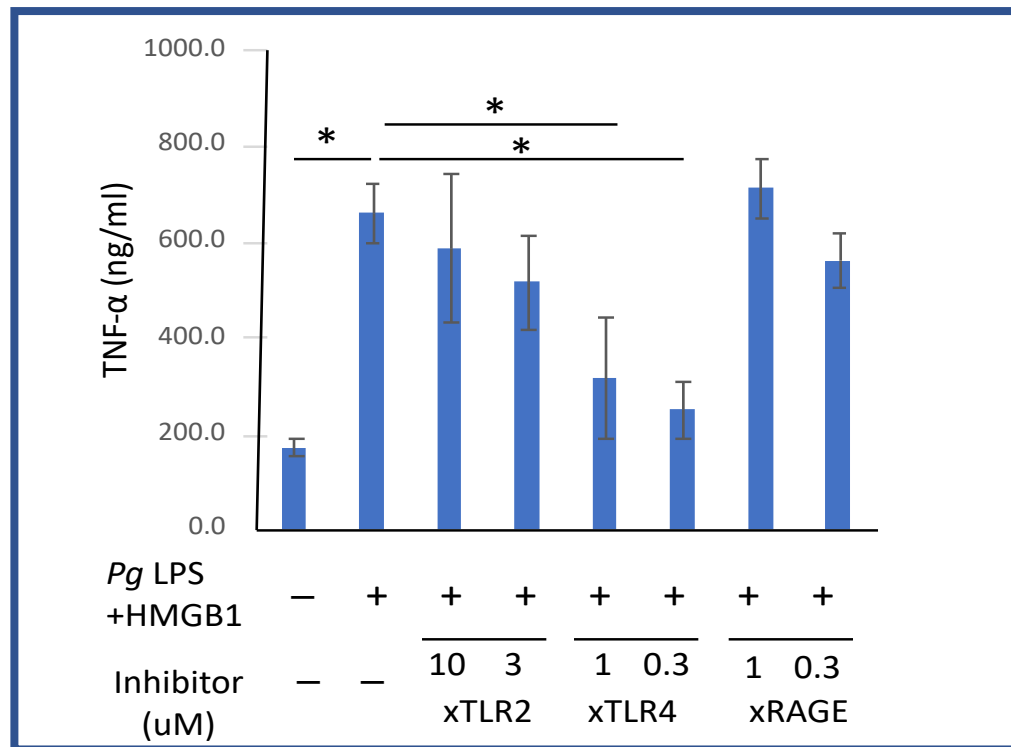


Figure 13: Effects of chemical inhibitors for TLR2, TLR4 and RAGE on TNF- α production by macrophages stimulated with *Pg*-LPS + HMGB1. TLR2 inhibitor: CU CPT 22 (Sigma), TL4 Inhibitor: TAK 242 (Sigma), RAGE Inhibitor: FPS-ZM1 (Sigma)

3.5. SUMMARY OF FINDINGS

- Combination of HMGB1 and *Pg*-LPS showed remarkable synergistic effects on TNF- α production by macrophages. At least 3-fold increase of TNF- α production was observed by HMGB1/*Pg*-LPS complex. However, such synergistic effects were not detected between *E. coli*-LPS and HMGB1 which showed a modest additive effect.
- Combination of HMGB1 and *Pg*-LPS also increased the proliferation of macrophages, whereas of HMGB1 alone, *Pg*-LPS alone, *E. coli*-LPS alone, or

the combination between *E. coli*-LPS and HMGB1 did not affect the proliferation of macrophages.

- Macrophages used in this study expressed mRNAs for TLR2, TLR4 and RAGE as putative ligands for HMGB1.
- Neither *Pg*-LPS or HMGB1 changed the expression levels of all three PAMP receptors expressed by macrophages.
- According to the assay using PAMP-inhibitors, HMGB1 appeared to bind both TLR2 and TLR4, while *Pg*-LPS acted on TLR4.
- The effect of HMGB1-*Pg*-LPS complex to promote TNF- α production was only inhibited by TLR4 inhibitor, but not TLR2 or RAGE inhibitor, suggesting that TLR4 play a key role in responding to HMGB1/*Pg*-LPS complex.

CHAPTER 4: DISCUSSION

Results from this study demonstrated that extracellular HMGB1 can form a hyper-inflammatory complex with *Pg*-LPS, while much lesser extent with *E. coli*-LPS, which, via ligation with TLR4, upregulates TNF- α production from macrophages. According to our results, at the same concentrations tested, the activities of *Pg*-LPS and HMGB1 to induce TNF- α production from macrophages were significantly lower than that of *E. coli*-LPS. Nonetheless, only *Pg*-LPS, but not *E. coli*-LPS, showed the remarkable synergistic effect with HMGB1 on TNF- α production by macrophages, suggesting that *Pg*-LPS possesses a distinct property to induce pro-inflammatory response working in concert with HMGB1. Furthermore, such a *Pg*-LPS/HMGB1-complex mediated promotion was only found in production of TNF- α , but not IL-6, whereas *Pg*-LPS/HMGB1 complex also upregulated the proliferation of macrophages. This study implicated that pathogenic engagement of opportunistic pathogen, *P. gingivalis*, may be elicited by locally elevated host-derived extracellular HMGB1 that can augment the activity of *Pg*-LPS to stimulate TLR4 for induction of TNF- α production.

Current paradigm of periodontitis supports that dysbiosis of the oral microbiome may play a crucial pathologic role in the onset and progression of this disease. It is thought that small number of key bacteria in the community drives the conversion of the healthy microbiome to dysbiosis state.²⁰ The emerging theory of “Keystone-Pathogen Hypothesis”²¹ proposes that certain low-

abundance opportunistic pathogens, represented by *P. gingivalis*, can cause dysbiosis by disrupting the host immune system and misshaping the microbiota in periodontal tissue. Although a mouse model of *P. gingivalis* induced periodontitis demonstrated that complement component 3 (C3) is associated with the periodontal pathogenesis that augments the level of inflammation,²² the precise molecular mechanism accounting for the *P. gingivalis*-mediated upregulation of proinflammatory response in human patients remains elusive. To this end, we propose that HMGB1/*Pg*-LPS complex may account for the dysbiosis caused in periodontal tissue with periodontitis. Accumulated lines of evidence support that inflammation is not induced by commensal Gram-negative bacteria that colonize in the mucosal tissue of host due to the mechanism of “endotoxin tolerance”.²³ In the tissue culture system, common LPS, represented by *E. coli* LPS, mitigates the pro-inflammatory response by host immune cells to the secondary exposure to the same LPS, illustrating the state of “endotoxin tolerance” in the in vitro context. However, it is reported that, *Pg*-LPS-exposed host cells do not render to the state of endotoxin tolerance,^{24,25} suggesting that unique structure of *Pg*-LPS¹⁴ also functions differently from the common LPS. Based on these lines of evidence and the results obtained from this study, it is theorized that locally released extracellular HMGB1 is engaged in pathogenic conversion of *Pg*-LPS in the periodontal tissue where abundantly available common LPS does not induce inflammation due to the endotoxin tolerance.

While it was reported that HMGB1 can generate a hyper-pro-inflammatory complex with *E. coli*-LPS that increases production of pro-inflammatory cytokines, including TNF- α , IL-6 and IL-8,^{6,19} our experimental model using RAW264.7 cells could not reproduce aforementioned results. Although HMGB1 by itself is weak in induction of inflammatory response, its interaction with TLRs²⁶ or RAGE²⁷ promotes the production of cytokines and other inflammatory molecules from innate immune cells. As noted in the introduction, HMGB1 also binds to DNA and LPS.²⁸ Recent studies identified other pro-inflammatory mediators, such as, thrombospondin, TREM-1, CD24, and CXCL12, can also work in concert with HMGB1 to upregulate inflammatory responses.^{28,29} Furthermore, three isoforms of HMGB1 were reported including 1) disulfide HMGB1 that can induce cytokine production via ligation with TLR4, 2) fully reduced HMGB1 that upregulate CXCL12-mediated chemotaxis by ligation with CXCR4, and 3) oxidized HMGB1 which is inactive in activating either TLR or CXCR4.³⁰ Therefore, the reason why *E. coli*-LPS/HMGB1 complex did not show the synergistic effects may be attributed to the presence of other factor produce by RAW264.7 cells that may hinders the ligation with TLR4 or oxidation of HMGB1 in the culture of RAW264.7 cells that attenuated the ligation of *E. coli*-LPS/HMGB1 complex with its receptor.

Recent reports have pointed out that HMGB1 is released in the gingival crevicular fluid of patients with periodontitis as well as peri-implant crevice fluid (PICF) of patients with peri-implantitis.^{9,31-33} The high level of HMGB1 expression

is found in the active sites of in GCF and PIFC in conjunction with increased levels of several pivotal pro-inflammatory cytokines, including TNF- α , IL1- β , IL-6 and IL-8, suggesting that HMGB1 and these pro-inflammatory cytokines may develop a positive feedback loop to promote inflammation in chronic periodontitis and peri-implantitis.³³ *In vitro* stimulation of gingival epithelial cells with TNF- α and IL-1 β increase their secretion of HMGB1.^{7,34} In the present study, since we focused on the effects of HMGB1 on the productions of proinflammatory cytokines from macrophages, the release of HMGB1 from those macrophages was not monitored. It is very intriguing to know whether positive feedback loop to promote inflammation can be elicited in the *in vitro* assay using RAW264.7 cells.

In the septic shock, level of HMGB1 increases later than the elevation of TNF- α or IL-1 β in the circulation, indicating the possible engagement of HMGB1 in the endotoxin-induced lethal damage of host.^{35,36} Indeed, in the mouse model of Cecal Ligation Puncture (CLP) or LPS-induced septic shock, the lethality and elevated production of proinflammatory cytokines in circulation is significantly suppressed by Dabrafenib that can attenuate the extracellular release of HMGB1³⁷ or by Glycyrrhizin, a direct HMGB1 antagonist.³⁸ Anti-HMGB1 neutralizing antibody administered to the mice induced of periodontitis by infection with *P. gingivalis* inhibits the local secretion of proinflammatory cytokines as well as bone resorption.³⁹ According to our results, chemical antagonist for TLR4 (TAK-242) inhibited the *Pg*-LPS/HMGB1-complex induced TNF- α production from macrophages. Thus, if our hypothesis is proven to be true

by future experiments using an animal model of periodontitis, it is expected that at least one of the above listed inhibitors for HMGB1 (Dabrafenib, Glycyrrhizin, xHMGB1-antibody or TAK-242) would show the clinical potency in the prevention and amelioration of periodontitis associated with *P. gingivalis*. Since thus far there is no small molecule-based drug is available for treatment of periodontitis, and the treatment of periodontitis is relay on the invasive procedures, the results obtained from current study may lead to the development of paradigm-shifting non-invasive therapeutic approach for periodontitis.

4.1 STUDY LIMITATIONS AND FUTURE STUDIES CONSIDERATIONS

We must be cautious not to rule out the possibility of the endogenous HMGB1 being released from RAW264.7 cells, which in turn could have promoted the cytokine productions directly without formation of complexes with other molecules. To test whether HMGB1 is released from RAW264.7 cells, future studies could add to their assessments the level of HMGB1 released into the culture supernatant following RAW264.7 cells stimulation with *P. gingivalis*-LPS or *E. coli*-LPS monitored using HMGB1 ELISA.

There is a possibility that the recombinant HMGB1 may not possess the biological property of naturally available HMGB1, which, in turn, impairs the function to form complex with bacterial LPS. A lack of biological activity of recombinant HMGB1 may be caused by misfolding of recombinant HMGB1 during the synthesis using *E. coli* expression system. It is not clear, among the three

reported forms of HMGB1, i.e., 1) disulfide HMGB1, 2) fully reduced HMGB1, and 3) oxidized HMGB1,³⁰ which form of HMGB1 is released in the inflamed tissue with periodontitis. Furthermore, the misfolding of recombinant HMGB1 could be detected by the unexpected outcome, i.e., the mixture of *E. coli* LPS and recombinant HMGB1 does not promote the inflammatory response by RAW264.7 cells. In this case, future studies should consider purify the endogenous HMGB1 from the blood of healthy blood donor using anti-HMGB1 affinity purification column. Then, the effects of purified authentic HMGB1 on *P. gingivalis* LPS could be compared by the macrophage responses from both recombinant and endogenous HMGB1 complexes.

The *in vitro* nature of this study might not reflect the physiological context of patient with periodontitis. However, the range of pro-inflammatory cytokine productions in response to stimulation with HMGB1-LPS should vary extensively among human individuals, while intra-individual differences can also be expected. For example, it is well established that the progression of periodontitis occurs in a site-specific manner in the same patient. To address this issue, the level of HMGB1/*Pg* LPS complex in patients gingival crevicular fluid (GCF) sampled from different teeth could be assessed in future studies, along with levels of *P. gingivalis* and pro-inflammatory cytokines.

CHAPTER 5: CONCLUSIONS

High mobility group box protein 1 (HMGB1) is a nuclear protein that can be released from host cells during infection or sterile injury, where it acts as a typical alarmin exerting proinflammatory effects either by itself or through interaction with endogenous or exogenous factors.^{40,41} Extracellularly released HMGB1 has the property to bind to not only DNA, but also pathogenic molecules, such as LPS, forming immune complexes that can trigger pro-inflammatory responses in a synergistic way and have been implicated in the pathogenesis of many immune and inflammatory diseases.⁴²⁻⁴⁴

This study demonstrated that HMGB1 can make a hyper inflammatory complex with *Pg*-LPS, but not *E. coli*-LPS, which in turn activates TLR4 and upregulates the production of TNF- α from macrophages. In addition to HMGB1 signaling activity to promote cytokine release, the combination of HMGB1 and *Pg*-LPS displayed the ability to increase the proliferation of macrophages. These results indicate that locally released HMGB1 may up-regulate the pathogenic engagement of keystone pathogen, *P. gingivalis*, in periodontitis.

Furthermore, the enhanced TNF- α production from macrophages induced by HMGB1 complex with *Pg*-LPS was inhibited by specific chemical antagonist for TLR4 (TAK 242). This finding suggests that the observed effects of HMGB1 and *Pg*-LPS induction of hyper-proinflammatory responses may be mediated through the reciprocal receptors of the HMGB1-partner molecules. Consequently, this

mechanism may lead to the development of a potential therapeutic approach to inhibit the disease progression of *P. gingivalis*-associated periodontitis.

Since periodontitis not only deteriorates the patient's quality of life, but also increases the risk for chronic systemic diseases,⁴⁵ there is a strong need for developing novel preventive and therapeutic approaches for this disease. Therefore, findings from the present study were paramount in the understanding of basic pathophysiology of HMGB1 as a pro-inflammatory promoter, as well as its role and mechanisms in the context of periodontitis. Moreover, the assessment and validation of HMGB1 receptors and inhibitory signaling molecules will bring us closer to their clinical application for designing a novel HMGB1-targeted therapeutic approach.

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